The effect of puberty on diurnal sodium regulation

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MATERIALS AND METHODS

Two major pathophysiological conditions, hypertension and enuresis, have been related to the timing of the sodium excretion during day and night. Renal sodium handling is complex and the regulation is dependent on several factors from arterial blood pressure and glomerular filtration rate (GFR) to specific sodium-regulating hormones with effects on renal tubular reabsorption (19). The major sodium-regulating hormones are the renin-angiotensin-aldosterone system (RAAS) that retain sodium and atrial natriuretic peptide (ANP), which increases sodium excretion (19). Sex differences in renal function are well established from human adult and animal studies with lower responsiveness of the RAAS in females compared with males (21). Both estradiols and testosterone have a direct modulating effect on sodium excretion and the RAAS system (24, 28). During the transition from childhood to adulthood major sex differences appear. This involves changes in body composition, blood pressure, and sex-hormone profile (1, 2, 4, 33), all of which could affect the regulation of sodium excretion.

In healthy children renal sodium handling is sparsely investigated and the circadian rhythm of sodium regulation hormones in relation to sodium excretion has not been studied. From previous studies it is known that there are decreasing morning plasma levels of renin (REN), angiotensin II (ANG II), and aldosterone (ALDO) throughout childhood, reaching adult levels postpuberty with no sex differences observed (6, 10, 14, 32, 37, 38). ANP is found to be independent of both age and sex in children with morning levels similar to the ANP levels in adults (41). A circadian rhythm of sodium-regulating hormones in children has been detected, but the influence of sex and puberty has not been described [ANP (29) and REN, ANG II, and ALDO (8, 17, 20, 25, 30)]. Investigating the regulation of sodium excretion in children might add to our understanding of hypertension and enuresis pathophysiology.

We designed a study to investigate renal sodium handling in children to answer the following questions: 1) Does sex or puberty stage affect sodium excretion. 2) Does sex or puberty stage affect some or several of the involved regulatory systems? 3) Can we confirm a circadian rhythm in childhood sodium regulation? Under standardized conditions in an in-patient setting we measured hemodynamic parameters, plasma levels of sodium-regulating hormones (ANP and RAAS), and sex hormones (testosterone and 17-β-estradiol) as well as urinary excretion of solutes in four different groups of children, each group defined by sex and puberty stage.

MATERIALS AND METHODS

The study protocol was approved by the regional Committee on Biomedical Research Ethics, and informed consent was obtained from all participants.

Study Subjects

Thirty-nine healthy volunteers were recruited from the local area. They were not patients followed in the department. Inclusion criteria for children in prepuberty were as follows: age 7–8 yr, Tanner stage 1 (girls: breast stage 1; boys: testicle volume 1–2 ml) and for children in puberty: age 12–15 yr, Tanner stage 3–4 (girls: breast stage 2–3 and premenarche; boys: testicle volume 8–15 ml). All the participants had
a normal physical examination including blood pressure measurement. Height and weight were within 2 SD of normal growth (43), and they also had complete bladder emptying upon voiding and normal dip-stick analyses. There was no history of day or night urinary or fecal incontinence after the age of 4 yr and no known prior severe illnesses or use of any medications, drugs, alcohol, or tobacco.

Study Design

The experimental procedure was a 24-h in-patient study under standardized conditions for comparison of diuresis and hormone profiles between sex and puberty stage groups.

Participants were admitted to the Department of Pediatrics 1 night before the study for accommodation to the environment. On the morning of study initiation an intravenous, heparinized catheter was inserted in a cubital vein for blood sampling. During the experimental procedure diet and fluid intake were standardized as directed by a pediatric dietitian. Sodium (3 mmol/kg body wt and water 25 ml/kg body wt divided in 2/3 before 1600 and 1/3 until bedtime). Meals were served at 0800, 1200, and 1800, and caffeine-containing beverages and additional servings were not allowed. Activity was allowed between 0800–2200 and bedtime was set to no later than 2200. Sleep during daytime was not allowed. The children were supervised by an adult during the entire experiment. At 0800 on the study day the experimental procedure began.

Blood samples were drawn every 4 h, participants were in bed and lay supine at least 15 min before each blood sampling, and during night care was taken not to disturb the children’s sleep. To prevent clotting of the cannula the catheter was flushed with 10 ml isotonic saline and 0.25 ml heparin (50 IE) was instilled. Blood samples from 12.00 h and the subsequent sampling time points were analyzed for plasma (P) concentrations of P-ANG II, ALDO, ANP, 17-β-estradiol, and testosterone.

Urine was fractionally collected in 4-h intervals during daytime, starting after bladder emptying at 0800. Nighttime urine collection started at 2200 until the end of the study at 0800. All voidings were spontaneous except after each blood sampling and before bedtime where the participants were asked to empty their bladder. The urine volume was measured and the concentration of sodium, potassium, as well as creatinine was determined.

Arterial blood pressure was noninvasively monitored every hour (ambulatory blood pressure monitor; Spacelab Model No. 90207).

A home recording with a 48-h urine collection was made during a weekend following the admission. The urine was refrigerated and delivered to the laboratory within 24 h after the end of the collection period. There were no restrictions regarding fluid or food intake, activity level, or hours of sleep.

Biochemistry Determinations

Sodium, potassium, and creatinine measurements were carried out at the Department of Clinical Biochemistry; plasma and urine analyses were performed on a Vitros 950 analyzer.

P-REN was measured in EDTA-plasma by a commercially available kit (DSL-25100, Active Coated-Tube IRMA; Diagnostic Systems Laboratories, Webster, TX) with a detection limit of 0.7 pg/ml. The interassay and intra-assay coefficient of variation was 2.64 and 1.63%, respectively.

P-ANG II was measured in plasma following ethanol extraction using a previously described RIA with modifications (18). The antibody was a rabbit anti-ANG II antibody (G225, produced by J. Danser, Dept. of Pharmacology, Erasmus University Medical Centre, Rotterdam, The Netherlands). The interassay and intra-assay coefficient of variation was 13.2 and 12.9%, respectively; the detection limit was 0.9 pg/ml.

P-ALDO was determined in EDTA-plasma using a commercially available RIA kit (DSL-8600, Active Aldosterone-Coated-Tube RIA; Diagnostic Systems Laboratories). Interassay and intra-assay coefficient of variation was 11.6 and 8.6%, respectively, with no cross reactivity with other natriuretic peptides.

17-β-Estradiol was measured in serum using a modified extraction-RIA assay, with a sensitivity of 4 pmol/l. The inter- and intra-assay coefficient of variation increased when close to the detection limit and was 5–19 and <17%, respectively (4).

Testosterone was measured in serum using a modification of a commercially available RIA, with a detection limit of 0.03 nmol/l and a coefficient of variation of 6–11% intra-assay and 5–15% interassay. 17-β-Estradiol and testosterone measurements were performed by the Pediatric Growth Research Center (Gothenburg, Sweden) (3).

Other Determinations

Residual urine was measured by ultrasound (BVI 2500+; Verathon). Urine dip-stick was performed using a Multistix 7 (Bayer Diagnostics).

Calculations

On the basis of urine and plasma measurements excretions (E) and clearance (C) were calculated for electrolytes and creatinine using standard formulas (E = UV, C = UV/PΔt, where U is urinary concentration, V is urine volume, P is the arithmetic mean of the plasma concentrations in the period and Δt is the duration of the period). The clearance of creatinine was used for GFR approximation after adjusting for body surface area (BSA). BSA = [weight (kg) × height (cm)/173.1]1/2 [Mosteller formula (23)]. Fractional excretions (FE) were defined according to the formula FEi(%) = (Ci/C-creatinine) × 100. Diuresis and filtered load (F) were defined as F = C-creatinine × P, and filtered load, excretions, and clearance were body weight adjusted.

Statistics

A group was defined by sex and puberty stage and association between parameters were tested for effect of group in the one-way ANOVA (nonrepeated measurements). Mean values between groups were tested using students t-test. Repeated measurements were tested in a mixed effect model (modified multivariate ANOVA for repeated measurements) for the effect of sex, puberty, and time. Differences between groups are given in mean percent with [95% coefficient intervals]. Circadian rhythm was defined as effect of time in the mixed effect model.

All analyses were performed using STATA10.0 software. Results are given as means ± SE. Statistical significance was defined by a P < 0.05.

RESULTS

Baseline characteristics of the participants are shown in Table 1. One prepuberty girl was excluded due to an episode of enuresis during admission. Data from the admission and home recordings were comparable both concerning “24-h sodium” and “24-h potassium” excretions, and there was no difference between groups.

The sex-hormone levels were as expected very low in the prepuberty group and with the highest levels of testosterone in puberty boys and the highest levels of 17-β-estradiol in puberty girls (Table 1).
Renal electrolyte handling. Sodium excretion, clearance, and fractional excretion showed a pronounced circadian rhythm with a considerable nighttime decrease (reduction from day to night: E-Na: 35% [23–46%]; C-Na: 35% [23–44%]; and FE-Na: 41% [30–50%]; Table 2). Filtered sodium displayed a reversed circadian rhythm with 10% [2–17%] nighttime increase. There was a nonsignificant trend towards lower excretion of sodium in girls (reduction: E-Na: 22% [1–42%]; $P = 0.059$), but sodium filtration, clearance and fractional excretion was similar in boys and girls. There was no impact of puberty stage on excretion, clearance, or fractional excretion of sodium but the filtration of sodium was significantly higher in the prepuberty group (F-Na pre vs. puberty: 26% [14–37%]; Table 2).

Potassium showed pronounced circadian rhythm in both excretion and clearance and fractional excretion with a marked nighttime decrease (E-K: 59% [53–100%]; C-K: 60% [54–100%]; and FE-K: from 9.7 ± 0.5 to 3.7 ± 0.3 mmol·kg$^{-1}$·h$^{-1}$). There was no impact of sex or puberty stage on excretion, clearance, or fractional excretion of potassium and no influence on the circadian changes.

As expected, diuresis decreased with a 50% [42–58%] reduction from day to night, and there was no impact of sex or puberty stage on urine volume or the circadian changes in urine output. Estimated GFR (eGFR) displayed a slight but significant circadian rhythm with a 10% [3–18%] increase during night. eGFRs were 15% [2–27%] lower in girls compared with boys, and eGFRs were 15% [2–26%] lower in puberty compared with prepuberty.

### Hemodynamics

Mean arterial blood pressure (MAP) displayed a circadian rhythm with lower nighttime levels in all groups (Fig. 1). Differences were found between groups in nighttime MAP ($P < 0.05$). Puberty girls had significantly higher nighttime MAP compared with prepuberty girls ($P < 0.05$). Otherwise no difference in MAP between groups was found. (Table 3) In all groups there was a circadian rhythm in heart rate with lower nighttime levels (Fig. 1). The puberty boys had significantly lower heart rate during both night and day compared with puberty girls (day: $P < 0.05$; night: $P < 0.001$).

### Table 1. Baseline characteristics of the participants

<table>
<thead>
<tr>
<th></th>
<th>Prepuberty</th>
<th>Puberty</th>
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<tbody>
<tr>
<td></td>
<td>Boys</td>
<td>Girls</td>
</tr>
<tr>
<td>Admission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>8.2 ± 0.5</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>28.1 ± 4.7</td>
<td>28.8 ± 3.9</td>
</tr>
<tr>
<td>Height, cm</td>
<td>131.4 ± 6.6</td>
<td>132.1 ± 4.2</td>
</tr>
<tr>
<td>Body surface area, m²</td>
<td>1.01 ± 0.11</td>
<td>1.03 ± 0.08</td>
</tr>
<tr>
<td>Breast stage</td>
<td>1</td>
<td>3 (2–3)</td>
</tr>
<tr>
<td>Testicle size, ml</td>
<td>2 (1–2)</td>
<td>12 (8–15)</td>
</tr>
<tr>
<td>U-Na, mol·kg body wt$^{-1}$·h$^{-1}$</td>
<td>2.82 ± 0.29</td>
<td>2.39 ± 0.37</td>
</tr>
<tr>
<td>17-β-Estradiol, pmol/l</td>
<td>5.0 (2–10)</td>
<td>9.5 (2–30)</td>
</tr>
<tr>
<td>U-K, mol·kg body wt$^{-1}$·h$^{-1}$</td>
<td>1.56 ± 0.13</td>
<td>1.36 ± 0.17</td>
</tr>
<tr>
<td>Testosterone, nmol/l</td>
<td>0.2 (0–0.4)</td>
<td>0.2 (0–0.5)</td>
</tr>
</tbody>
</table>
| Values are means ± SD, except when breast stage and testicle size and then median and range are given. U, urine; Na, sodium; K, potassium. *Differences between groups defined by sex and puberty stage; †differences between groups in daytime mean; ‡differences between “inpatients” and “outpatients”; NS, nonsignificant.

### Table 2. Urine output parameters presented as day and night values in groups defined by sex and puberty stage

<table>
<thead>
<tr>
<th></th>
<th>Prepuberty</th>
<th>Midpuberty</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boys</td>
<td>Girls</td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td>Night</td>
</tr>
<tr>
<td>Uflow, ml·kg$^{-1}$·h$^{-1}$</td>
<td>1.74 ± 0.15</td>
<td>1.04 ± 0.13</td>
</tr>
<tr>
<td>GFR, ml·min$^{-1}$·1.73 m$^{-2}$</td>
<td>204 ± 31</td>
<td>225 ± 28</td>
</tr>
<tr>
<td>$F_{\text{Na}}$, mmol·kg$^{-1}$·h$^{-1}$</td>
<td>36 ± 6</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>$C_{\text{Na}}$, mmol·kg$^{-1}$·h$^{-1}$</td>
<td>0.13 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>$F_{\text{K}}$, %</td>
<td>0.95 ± 0.10</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td>$C_{\text{K}}$, mmol·kg$^{-1}$·h$^{-1}$</td>
<td>0.46 ± 0.07</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>$E_{\text{Na}}$, mmol·kg$^{-1}$·h$^{-1}$</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>$C_{\text{K}}$, mmol·kg$^{-1}$·h$^{-1}$</td>
<td>19.8 ± 1.9</td>
<td>10.2 ± 1.2</td>
</tr>
<tr>
<td>$F_{\text{K}}$, %</td>
<td>9.1 ± 1.4</td>
<td>3.8 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. Uflow, urine excretion rate; GFR, glomerular filtration rate; $F_{\text{Na}}$, filtered Na load; $E_{\text{Na}}$, Na excretion; $C_{\text{Na}}$, Na clearance; $F_{\text{K}}$, fractional K excretion. Statistical differences between groups are stated under statistics; results are from a mixed effect model except when marked with a symbol: *differences between groups in daytime mean; †differences between groups in nighttime mean; NS, nonsignificant.
0.01) and during daytime compared with prepuberty boys (P < 0.05; Table 3).

**Sodium-regulating hormones.** All hormones displayed significant circadian rhythm (Fig. 2). The timing of the changes did not differ between boys and girls or between puberty groups, but the level of both P-REN and P-ANG II was highly dependent on puberty stage. In P-REN there was no difference between boys and girls but a significant effect of puberty stage with 25% [3– 42%] lower levels in the puberty group. Also, the level of P-ANG II showed no differences between boys and girls but a significant effect of puberty stage with 28% [6– 46%] reduction in plasma P-ANG II levels in the puberty groups was observed.

In both the level of P-ALDO and the level of P-ANP, there was no effect of either sex or puberty stage.

We found a slight but significant negative association between P-ANG II and E-Na \( \left[ -0.282 \right] \) \( \alpha \), P < 0.01. Again there was no difference between sex and puberty groups (P = 0.253), but the puberty boy group had lower P-ANG II (P < 0.001). Increased MAP was associated with decreased levels of ANG II with a significant negative correlation between MAP and ANG II levels \( \left[ -0.227 \right] \) \( \alpha \), P < 0.001) and no difference between sex or puberty groups (P = 0.394). Again the puberty boy group had significantly lower P-ANG II levels (P < 0.01).

**Plasma electrolytes.** P-Na and P-K were stable during the observation period, and no circadian rhythm was detected in either of the electrolytes. Sex had no effect on P-Na but significantly lower P-Na levels were observed prepuberty compared with puberty. P-K levels were independent of both sex and puberty stage (Fig. 3).

**DISCUSSION**

This study demonstrates that sodium regulation in 7- to 15-yr-old boys and girls express a marked circadian rhythm. We observed a nighttime decrease in sodium excretion and blood pressure concurrent with an increase in plasma levels of RAAS and ANP. The timing of the changes was consistent in all children, but several of the parameters were affected by sex and puberty stage. The main findings of this study were that boys and girls have similar circadian sodium regulation but during puberty a different set point for sodium regulation evolves with reduced filtration of sodium in conjunction with decreased levels of sodium-reabsorbing hormones (P-ANG II and P-REN) resulting in unchanged sodium excretion.

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**Table 3. Diurnal blood pressure and heart rate in groups defined by sex and puberty stage**

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>HR, min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Night</td>
</tr>
<tr>
<td>Prepuberty</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>82 ± 5</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>Girls</td>
<td>81 ± 6</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>Puberty</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>82 ± 5</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>Girls</td>
<td>84 ± 7</td>
<td>77 ± 8*</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial blood pressure; HR, heart rate. Statistical differences between groups using one-way ANOVA: *differences between sex; †differences between puberty stages; NS, nonsignificant.
Differences Between Boys and Girls

Male and female differences in sodium regulation and especially RAAS have been intensely explored to explain the sex differences in hypertension and progression in renal disease (22). Most studies find lower blood pressure (42) and higher RAAS levels (22, 36) in women, but when comparing the renal sodium excretion there are no sex differences (11). This study confirms the uniform sodium excretion in boys and girls; however, in contrast to studies in adults, we observe similar levels in all sodium-regulating hormones and blood pressure between the sexes. From this we conclude that the development of significant gender-specific differences must occur later than the puberty phase. Although gender differences in circulating RAAS hormone levels and blood pressure have not yet developed, the renal sodium handling was affected. Despite the significantly lower GFR in girls, there was only a tendency towards lower filtered sodium load and sodium excretion. It seems possible that the blood pressure-GFR relationship under normal circumstances is affected by gender-specific regulation already during childhood although no significant differences in circulating RAAS-hormone levels are to be found. This could be explained by a gender-specific expression of receptors or secondary intracellular messengers in the RAAS system. Animal studies find a lower ratio of angiotensin type 1 (AT1) to angiotensin type 2 (AT2) receptors in female compared with male rats (34) and the difference in receptors is thought to explain why female compared with male rats can excrete the...
same amount of sodium at lower systemic blood pressure (13). Other components of the RAAS system, as for example ANG(1–7), have been identified. These components have been found to express gender difference in the effect on blood pressure and disease progression (35). A modulating effect on the sex hormone levels has, to our knowledge, not been found. Linkage studies have pointed towards a genetic variation in the ACE gene that influences the blood pressure in a sex-specific manner (9). However, until now most studies of gender differences in renal function have found a modulating effect of sex-hormone levels (26, 34).

The Impact of Puberty

In prepuberty, both male and female sex hormone levels are low but during puberty there is a marked increase in sex hormone levels in both sexes, as seen in the present study (1, 2, 4, 33). Testosterone is thought to change the pressure-natriuresis relationship and explain the male-female differences in RAAS in hypertensive Dahl-rats (27). On the other hand, estrogens have multiple effects on sodium regulation. Thus evaluation of RAAS in normal menstrual cycling women and during estrogen replacement therapy has revealed activation of RAAS in the presence of high estrogen levels (7). In our study, sodium excretion was not affected by puberty stage, but significant differences in renal sodium regulatory mechanisms between prepuberty and puberty groups of children were observed. Plasma sodium levels were higher but sodium-retaining hormones ANG II and REN levels were lower in puberty compared with prepuberty. These findings suggest that a different set point for the sodium balance is evolving during early puberty. It is generally accepted that there are decreasing levels of RAAS with increasing age during childhood (5). The renal handling of sodium was affected by puberty with significantly higher filtration of sodium in prepubertal children despite lower plasma sodium levels. This reflects differences between groups in GFR with lower GFR during puberty. Data on GFR values in healthy children are sparse, but our observation of lower GFR in girls and during puberty is comparable to previously reported age and sex differences in children (40). A more pronounced activation of sodium-retaining hormones in prepuberty might compensate for the higher filtered sodium load. The differences in GFR do not seem to result from differences in blood pressure between puberty groups. The expected difference in blood pressure between 7 to 8 yr olds and 12 to 15 yr olds is only 2–3 mmHg (12, 39), and the variability in blood pressure within groups (6–7 mmHg) may overshadow the differences between groups. Both systolic and diastolic measurements are comparable to reference values for age and sex (12). The higher filtered sodium values and more pronounced RAAS activation in prepuberty compared with puberty are of interest for the pediatric population. They imply a physiological difference in sodium and blood pressure regulation between adults and children. For practical reasons studies of healthy children are sparse and the possibility of intervention is limited by ethical considerations.

This study has limitations due to the number of children included and the small changes observed in normal circadian regulation; future studies of the effect of sodium loading and ACE inhibition could, however, be performed and might elaborate our findings of pre- to puberty changes in sodium filtration and RAAS function.

We demonstrate a nighttime increase in the sodium-depleting hormone ANP. The ANP levels were only elevated at midnight at the first blood sampling 2 h after assuming the recumbent position. This might be an effect of the increase in preload due to change in posture (15) and the timing of the peak is in accordance with results from studies with similar designs (17, 29). We can conclude that both boys and girls, independent of puberty stage, have comparable levels of ANP and a midnight ANP peak. The entire nighttime urine output was collected in the morning; therefore, it is not possible from this study to analyze if the midnight ANP peak has an immediate effect on sodium excretion. Future studies are needed to clarify if ANP is a physiologically important factor in early nighttime sodium excretion.

Hypertension and nocturnal polyuria are two major pathophysiologic conditions where interrupted circadian rhythm in blood pressure regulation and sodium excretion is involved. Previous studies by this group have shown that sleep deprivation can increase nocturnal sodium excretion and reduce nocturnal blood pressure dipping (16, 20). Adult males are more sensitive to the effect of sleep deprivation than females, but no gender differences among 8 to 12 yr olds were found. This study shows that from the age of 7 yr both boys and girls have a clear and significant circadian rhythm in sodium excretion, highly dependent on increased nocturnal sodium reabsorption. There is a pronounced circadian rhythm with nighttime increase in REN and ANG II, the major sodium-preserving hormones. In the light of this study’s findings of an effect of puberty on sodium regulation, this has to be taken into account in the planning of future studies where interventions with both nondipping hypertension and nocturnal sodium excretion are planned.

Conclusion

This study is the first to demonstrate circadian changes in sodium regulation in children before and in puberty. Boys and girls initially have a similar circadian renal sodium excretion, but during puberty the renal handling of sodium changes, with a reduction in filtered sodium and a decrease in the level of P-ANG II and P-REN without changing the amount of sodium excreted. The findings of this study add to our better understanding of the renal sodium handling in healthy children and the impact of sex and puberty. This knowledge enables us to study aberrations in renal sodium handling, as these are evident in enuresis nocturna and hypertension, two clinical conditions with clear male overrepresentation.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: B.T.M., K.K., C.A.-L., J.C.D., and S.R. conception and design of research; B.T.M., K.K., and C.A.-L. performed experiments; B.T.M. and K.K. analyzed data; B.T.M., K.K., C.A.-L., J.C.D., and S.R. interpreted results of experiments; B.T.M. prepared figures; B.T.M. drafted the manuscript.
manuscript; B.T.M., K.K., C.A.-L., J.C.D., and S.R. approved final version of manuscript.

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